UNIVERSITI PUTRA MALAYSIA

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

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September 2002

Chairman: **Professor Abdul Manaf Ali, Ph.D.**

Faculty: **Food Science and Biotechnology**

A bio-assay guided approach was used to isolate photosensitizing compounds from *Typhonium flagelliforme Schott*, a plant considered by local communities to possess anticancer properties. Two putative photosensitizers, pheophorbide-a and a possible novel derivative, 13-2, 17-dihydroxyethyl pheophorbide-a were isolated using column chromatography and purified on thin layer chromatography under low light conditions. The phototoxic effect of pheophorbide-a and 13-2, 17-dihydroxyethyl pheophorbide-a on the growth of a variety of human cell lines was tested using the MTT assay. Both photosensitizers were inactive in the dark but when activated by light, mediated cell killing with IC_{50} values ranging from 0.12 to more than 4 μg/ml, with the leukemic cells being the most sensitive. Photodynamic cell killing was dependent on photosensitizer concentration, irradiation dose and drug incubation time. Preliminary characterization showed that both compounds tended to aggregate in aqueous solution and the aggregation was augmented by the presence of serum proteins. This correlated to a decrease in cytotoxic efficacy as the amount of serum in the media was increased suggesting that binding to serum proteins reduced drug

uptake and possibly altered intracellular localization patterns. It has been shown that photosensitizers mediate cell-killing through a free radical (Type I) or singlet oxygen (Type II) dependent reaction. Indirect tests showed that the major species generated by photoactivation using pheophorbide-a and 13-2, 17-dihydroxyethyl pheophorbide-a was singlet oxygen molecules with a minor contribution by other radical species. Furthermore, confocal laser scanning microscopy showed that these fluorescent photosensitizers accumulated in lysosomes, suggesting that the release of hydrolytic enzymes may be a common mechanism leading to necrotic injury. However, whereas some cell lines also showed intense membrane and cytoplasmic staining, others showed some mitochondrial accumulation, suggesting that other mechanisms may also contribute to cell-killing. Commercially available PDT compounds, including hypericin, appear to kill cells by inducing apoptosis. The treatment of HL60 cells with pheophorbide-a and 13-2, 17-dihydroxyethyl pheophorbide-a resulted in the appearance of typical apoptotic morphology, including membrane blebbing, apoptotic bodies, cell shrinkage and DNA-laddering, suggesting that these compounds also induce apoptosis. Singlet oxygen is thought to be the primary stimulus driving the induction of apoptosis and this was studied by using the singlet oxygen quencher sodium azide. The caspase family of proteases, which are inhibited by the peptide Z-VAD-FMK, are regarded as the main effectors of apoptosis. Zinc ions are inhibitors of endonucleases that cause apoptotic DNA fragmentation. We found that both sodium azide and Z-VAD-FMK effectively reduced the incidence of apoptosis induced by pheophorbide-a and 13-2, 17-dihydroxyethyl pheophorbide-a. Zinc did not affect 13-2, 17-dihydroxyethyl pheophorbide-a induced apoptosis but reduced pheophorbide-a induced apoptosis. Cycloheximide, a protein synthesis inhibitor, did not decrease the incidence of apoptosis for either photosensitizer but

actually increased 13-2, 17-dihydroxyethyl pheophorbide-a induced apoptosis. These results support the role of singlet oxygen as primary inducers and caspases as effectors of apoptosis. PDT induced apoptosis does not require the synthesis of new proteins but PDT may, in the case of 13-2, 17-dihydroxyethyl pheophorbide-a, induce the synthesis of proteins that protect cells from further oxidative damage. The results also suggest that PDT with 13-2, 17-dihydroxyethyl pheophorbide-a may activate an endonuclease that is not inhibited by zinc. The progression of apoptosis is highly regulated by pro-(Bax) and anti- apoptotic Bcl-2 family of proteins (Bcl-2, Bcl-X_L). Qualitative detection of these apoptotic marker proteins at time intervals of 4, 8, 12 and 24 hours showed that the ratios of Bax to Bcl-2 and of Bax to Bcl-X_L were markedly increased from 12 hours onwards and this, as has been suggested earlier, is a marker for apoptotic progression. However, because apoptosis can be detected as early as 2 hours post irradiation, Bcl-2 proteins may not play a major role in the initial induction of apoptosis but impacts apoptosis at a much later stage, possibly to ensure the complete demise of the cell. In conclusion, pheophorbide-a and 13-2, 17-dihydroxyethyl pheophorbide-a are effective photosensitizing compounds that kill cells mainly through apoptosis. The possibility of different apoptotic pathways being induced, especially by 13-2, 17-dihydroxyethyl pheophorbide-a, makes the development of these compounds as potential clinical drugs, an exciting prospect.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
untuk memenuhi keperluan untuk ijazah Doktor Falsafah

PENYIFATAN DUA BAHAN FOTOSENSITIZING DARI SPESIS *TYPHONIUM FLAGELLIFORME* SCHOTT DAN MEKANISME MENYEBABKAN KEMATIAN SEL KANSER

Oleh

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September 2002

Pengerusi: **Profesor Abdul Manaf Ali, Ph.D.**

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Teknik pengasingan biocerakinan berpandu digunakan untuk mengasingkan bahan fotosensitizing dari spesis *Typhonium flagelliforme Schott.* Dua fotosensitizer, pheophorbide-a dan 13-2, 17-dihydroxyethyl pheophorbide-a, satu bahan terbitan baru, diasingkan dengan menggunakan kromatografi kolum dan TLC di bawah keadaan cahaya rendah. Kesan fototoksik pheophorbide-a dan 13-2, 17-dihydroxyethyl pheophorbide-a ke atas sel-sel kanser manusia diuji dengan kaedah MTT. Kedua-dua fotosensitizer tidak berkesan dalam keadaan gelap tetapi berjaya membunuh sel dengan IC_{50} dari 0.12 hingga lebih 4 μg/ml, dan sel leukemia adalah paling sensitif. Pembunuhan secara fotodinamik bergantung kepada kepekatan fotosensitizer, dos penyinaran dan tempoh pengeraman dengan fotosensitizer. Pencirian awal menunjukkan bahawa kedua-dua kompoun berkumpul bila dilarut dalam larutan akueus dan pengumpulan bertambah dalam kehadiran protein serum. Sifat ini berhubung kait dengan kebolehan serum untuk mengurangkan kesan sitotoksik. Ini mungkin disebabkan serum dapat mengurangkan penyerapan kompoun ke-dalam sel dan juga mengubah lokasi kompoun di dalam sel. Fotosensitizer dapat membunuh sel melalui reaksi bahan radical bebas (Jenis I) atau

'singlet' oksigen (Jenis II). Kajian secara tidak langsung menunjukkan bahawa bahan utama yang diterbitkan melalui pengaktifan sinaran fotosensitizer pheophorbide-a dan 13-2, 17-dihydroxyethyl pheophorbide-a adalah singlet oksigen dengan penyumbangan yang kurang dari radikal lain. Kaedah mikroskop laser konfokal menunjukkan bahawa fotosensitizer bertempat di dalam organel lysosome dan ini mencadangkan bahawa pembebasan enzim hidrolitik adalah mekanisma yang digunakan untuk membunuh sel. Fotosensitizer juga dikesan pada membran sel, sitoplasma dan mitokondria. Ini menunjukkan bahawa mekanisma lain juga digunakan untuk membunuh sel. Fotosensitizer komersial, termasuk kompoun hypericin, didapati membunuh sel secara 'apoptosis'. Rawatan sel HL60 dengan pheophorbide-a dan 13-2, 17-dihydroxyethyl pheophorbide-a menghasilkan morfologi 'apoptotic' dan ini termasuk tompok-tompok membran, badan 'apoptotic', pengecutan sel dan fenomena tangga DNA. Singlet oksigen dianggap sebagai rangsangan primer untuk menyebabkan 'apoptosis' dan ini dikaji dengan menggunakan Natrium Azide, salah satu penyah singlet oksigen. Keluarga 'caspase' adalah effektor utama dalam proses 'apoptosis' dan aktiviti mereka dapat disekat oleh bahan peptida Z-VAD-FMK. Aktiviti endonukleas yang menyebabkan fenomena tangga DNA pula, dapat disekat oleh ion-ion Zink. Kami mendapati bahawa Natrium Azide dan Z-VAD-FMK dapat mengurangkan kejadian 'apoptosis' yang disebabkan oleh pheophorbide-a dan 13-2, 17-dihydroxyethyl pheophorbide-a. Zink tidak berkesan ke atas activity 13-2, 17-dihydroxyethyl pheophorbide-a tetapi dapat mengurangkan 'apoptosis' yang disebabkan oleh pheophorbide-a. Sikloheksimid, sesuatu bahan yang boleh menyekat sintesis protein tidak dapat mengurangkan kejadian 'apoptosis' tetapi sebaliknya 'apoptosis' yang disebabkan oleh 13-2, 17-dihydroxyethyl pheophorbide-a bertambah banyak. Hasil-hasil kajian ini

menyokong pendapat bahawa singlet oksigen adalah rangsangan primer dan enzim-enzim 'caspase' adalah efektor utama dalam proses 'apoptosis'. 'Apoptosis' yang disebabkan oleh terapi fotodinamik tidak memerlukan sintesis protein baru tetapi, terapi ini mungkin merangsangkan sintesis protein yang melindungi sel dari kerosakan yang melanjut seperti yang didapati dengan 13-2, 17-dihydroxyethyl pheophorbide-a. Hasil kajian juga mencadangkan bahawa terapi fotodinamik dengan 13-2, 17-dihydroxyethyl pheophorbide-a menghasilkan endonukleas yang tidak dapat disekat oleh ion zink. Kemajuan proses 'apoptosis' dikawal ketat oleh protein keluarga Bcl-2 yang menyokong (Bax) dan yang menentang (Bcl-2, Bcl-XL) proses ini. Kajian pengesanan kualitatif penanda protein 'apoptosis' pada masa 4, 8, 12 dan 24 jam selepas terapi fotodinamik menunjukkan bahawa nisbah Bax kepada Bcl-2 dan Bax kepada Bcl-XL semakin tinggi dari 12 jam selepas terapi. Ini, sebagaimana yang dianggap terdahulu, adalah penanda yang tepat untuk mengesan kemajuan proses 'apoptosis'. Walaubagaimanapun, 'apoptosis' dapat dikesan seawal 2 jam selepas rawatan dan ini mencadangkan bahawa keluarga Bcl-2 tidak memainkan peranan penting dalam proses induksi 'apoptosis'. Malah, keluarga protin ini lebih menyumbangkan kesan pada peringkat yang lebih lewat, mungkin untuk memastikan kematian sel tersebut. Sebagai kata-kata pengakhiran, pheophorbide-a dan 13-2, 17-dihydroxyethyl pheophorbide-a adalah fotosensitizer yang berkesan dan mereka membunuh sel secara 'apoptosis'. Fotosensitizer yang diperolehi, terutamanya 13-2, 17-dihydroxyethyl pheophorbide-a, didapati merangsangkan pelbagai cara yang berlainan untuk menyebabkan 'apoptosis'. Perkembangan fotosensitizer ini sebagai ubat klinikal merupakan satu prospek yang amat menarik.

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I certify that an Examination Committee met on the 3rd of September 2002 to conduct the final examination of Anthony Ho Siong Hock on his Doctor of Philosophy thesis entitled "Characterisation of Two Photosensitizing Compounds from *Typhonium flagelliforme* Schott and their Mode of Action in Inducing Cancer Cell Death" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

ANTHONY HO SIONG HOCK

Date: 19 NOV 2002

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$^1O_2$	:	Singlet oxygen
AIDS	:	Acquired immune deficiency syndrome
AIF	:	Apoptosis inducing factor
AMP	:	Adenosine monophosphate
ANT	:	Adenine nucleotide translocator
AO	:	Acridine orange
BH	:	Bcl-2 homology domains
BPD-MA	:	Benzoporphyrin derivative monoacid ring A
CAD	:	Caspase activated deoxyribonuclease
CARD	:	Caspase recruitment domain
CC	:	Column chromatography
Cdk	:	Cyclin dependent kinase
CO_2	:	Carbon dioxide
COSY	:	Correlated spectroscopy
DMSO	:	Dimethyl sulfoxide
ESI MS	:	Electro Spray Ionization mass spectrometry
FADD	:	Fas associated death domain
FBS	:	Foetal bovine serum
FITC	:	Flouresceinthiocynate
FT-IR	:	Fourier transform infrared spectroscopy
GM-CSF	:	Granulocyte macrophage colony stimulating factor
HMW	:	High molecular weight
HPD	:	Hematoporphyrin derivative
IAP	:	Inhibitors of apoptosis protein
ICS	:	Intersystem crossing
IFN	:	Interferon
IL	:	Interleukin
IMS	:	Intermembrane space
J	:	Coupling constant
LDL	:	Low density lipoprotein
m/z	:	Mass-to-charge-ratio
M	:	Molar
mA	:	Milliampere
mg	:	Miligram
min	:	Minutes
ml	:	Mililiter
MPT	:	Mitochondrial permeability transition
MS	:	Mass spectrum
NLS	:	Nuclear localization signal
nm	:	Nanometer
NMR	:	Nuclear magnetic resonance
PARP	:	Poly(ADP)ribose polymerase
PBS	:	Phosphate buffered saline
PDT	:	Photodynamic Therapy
PI	:	Propidium Iodide
PKA	:	Protein kinase A
PKC	:	Protein kinase C

PMSF	:	Phenylmethysulfonyl fluoride
PS	:	Phosphatidylserine
PTLC	:	Preparative thin layer chromatography
ROS	:	Reactive oxygen species
SDS	:	Sodium dodecyl sulphate
SOD	:	Superoxide dismutase
TEMED	:	N,N,N',N'-tetramethylenediamine
TRADD	:	TNFR1 associated death domain
Tris	:	Tris (hydrosylmethyl) aminoethane
TM	:	Transmembrane
TMS	:	Tetramethylsilane
TNF	:	Tumor Necrosis Factor
TLC	:	Thin layer chromatography
UV	:	Ultraviolet
VDAC	:	Voltage dependent anion channel
μ	:	Micro
%	:	Percentage
δ	:	Chemical shift
λ_{max}	:	In UV spectroscopy, the wavelength at which maximum absorption occurs

CHAPTER I

INTRODUCTION

Photodynamic therapy (PDT) is a relatively selective method for the destruction of localised solid tumors. Photosensitizers capture the energy from light, producing singlet oxygen and other free radicals that destroy the targeted tissue. Until a decade ago, tumour destruction was thought to occur primarily via cellular necrosis, those of the tumour cells and that of the accompanying vascular supply (Oschner, 1997). The discovery that PDT induced apoptotic cell death raised many questions regarding the mechanisms employed by the affected cell upon PDT (Agarwal, 1991). Many groups have now established the occurrence of apoptosis in parallel with necrosis in both *in vitro* and *in vivo* model systems (Ahmad and Mukhtar, 2000; Dougherty et al, 1998). The distinction of the type of predominant cell death observed is largely dependent on photosensitizer type, intracellular localization, experimental protocol and tumour type (Hassan and Mukhtar, 2000; Agostiniz, *et al.*, 2000). As yet, no universal mechanism of apoptosis can be proposed for PDT because the type of apoptotic pathway affected is dependent on the variables above as well.

Our understanding of the role of mitochondria in apoptosis (Adrain and Martin, 2001) has illuminated a possible unifying starting point for PDT induced apoptosis. Kessel and Luo (1998) showed that photosensitizers that localize in the